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TITLE: Mitochondrial-Based Treatments that Prevent Post-Traumatic Osteoarthritis in a Translational Large Animal Intraarticular Fracture Survival Model

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14. ABSTRACT

The results of Specific Aims 1 and 2 in the rabbit model strongly supported choosing amobarbital and NAC for testing in the porcine intra-articular fracture model, which was proposed for Specific Aim 3. Data from Specific Aim 3 showed that acute treatment with amobarbital or NAC had significantly delayed the onset of arthritis in a clinically realistic model of PTOA.

15. SUBJECT TERMS

post-traumatic osteoarthritis, large animal model, oxidative stress, mitochondria, mechanotransduction, amobarbital, n-acetyl cysteine

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1. Introduction

The purpose of this research is to investigate a novel therapeutic approach to prevent PTOA by treating mitochondrial dysfunction in chondrocytes resulting from intra-articular injury. We have shown that scavenging excessive injury-related mitochondrial oxidants, or preventing their excessive formation after a severe impact injury, prevented chondrocyte death and metabolic dysfunction in an osteochondral explant model [1-4]. Subsequently, we demonstrated that oxidant production is strain–dependent and that physiologic levels of mitochondrial oxidants generated in explants subjected to normal loads, were important promoters of chondrocyte ATP synthesis[5,6]. These findings are the basis of the treatment strategies pursued in this *in vivo* investigation.

2. Keywords

post-traumatic osteoarthritis, large animal model, oxidative stress, mitochondria, mechanotransduction, amobarbital, n-acetyl cysteine

3. Overall Project Summary

We reported previously that experimental work for Specific Aims 1 and 2 was finished and that the results strongly supported choosing amobarbital and NAC for testing in Specific Aim 3. The data showing the short-term chondroprotective effects of these drugs in the rabbit model are a key component of a comprehensive manuscript we have drafted that includes long-term results from the minipig intra-articular fracture (IAF) model from Aim 3. We recently completed all data collection and analysis for this aim (*Determine the efficacy of treatments that prevent ROS overproduction, scavenge ROS, or dissolve the cytoskeleton in mitochondria on preventing PTOA in a large animal IAF survival model*).

Specific Aim 1. Measure changes in chondrocyte ATP production, chondrocyte viability, and PTOA in a survival rabbit model of cartilage injury treated with electron transport complex I inhibition and ROS scavenging.

Task 1: Surgical injury to the rabbit medial femoral condyle (Months 1-36)

A. Experimental groups (N = 6/group; 6 animals sacrificed at 7 days, 6 animals treated with best treatment sacrificed at 42 days, and at 180 days post-op; 24 rabbits total)

1. Amobarbital (complex I inhibitor)
2. N-Acetylcysteine (ROS scavenger)

B. Control groups (N = 18/group; 6 animals sacrificed at each 7 days, 42 days, and 180 days post op; 42 rabbits total)

1. Impact control (rabbits receive injury but no treatment)
2. Sham control (rabbits receive surgery but no injury or treatment)
3. Normal control (no surgery or treatment; only 6 rabbits in this group)

Task 2: Confocal, Biochemical, and Histologic Analyses (Months 1-36)

A. Confocal imaging

1. Chondrocyte viability determined by Calcein-AM staining. Live cell density will be calculated.
2. Images analyzed with in-house software for cell counts

B. Biochemical Analysis

1. ATP production determined with assay of cartilage directly

C. Histologic Analysis

1. Safranin O staining to determine degeneration scores (HHGS scale of Mankin)
2. Immunohistology to determine MMP-3, MMP-13

Specific Aim 2. Measure changes in chondrocyte ATP production, chondrocyte viability, and PTOA in a survival rabbit model of cartilage injury treated with compounds that dissolve filamentous actin and microtubulin.

Task 1: Surgical injury to the rabbit medial femoral condyle (Months 1-36)

A. Experimental Groups (N = 6/group; 6 animals sacrificed at 7 days, 6 animals treated with the best treatment sacrificed at 42 days, and at 180 days post-op; 24 rabbits total)

1. Cytochalasin B
2. Nocodazole

B. Control Groups: same animals used in Specific Aim 1 for Impact Control, Sham Control, and Uninjured Control

Task 2: Confocal, Biochemical, and Histologic Analyses (Months 1-36). This is identical to Specific Aim 1.

Specific Aim 3: Determine the efficacy of treatments that prevent ROS overproduction, scavenge ROS, or dissolve the cytoskeleton in mitochondria on preventing PTOA in a large animal IAF survival model.

Task 1: Surgical creation of a physiologic realistic intraarticular fracture in the minipig specimens (Months 25 – 48; 48 pigs total)

A. Experimental Groups (12 animals/group; six animals sacrificed at 6 months and six animals sacrificed at 12 months; 24 pigs total)

1. Treatment one
2. Treatment two

B. Additional Control Groups (Months 25 – 48)

1. Injured Control: 12 animals (six sacrificed at 6 months and six sacrificed at 12 months after surgery)
2. Sham Control: (No impact injury, but hardware placement): 12 animals (six sacrificed at 6 months and six sacrificed at 12 months after surgery)

Safranin-O-stained sagittal sections through loaded areas of pig tali and tibiae were scanned and analyzed using a computer algorithm to assess cartilage degeneration on the Mankin scale (Figure 1). This revealed significant therapeutic effects of NAC and amobarbital at 6 months, and a trend toward benefit at 12 months. The algorithm was also used to assess zonal Safranin-O staining intensity at 6 months, which revealed that amobarbital and NAC had proteoglycan-sparing effects, particularly in the transitional and radial zones (Figure 2). These data suggested that matrix biosynthesis was preserved in treated joints. Close examination of chondrocyte oxygen consumption rate (OCR) at 6 months indicated that cells from untreated fractured limbs were hyper-respiratory, a phenotype characteristic of osteoarthritic chondrocytes. In contrast, chondrocytes from amobarbital or NAC treated joints respired normally (Figure 3), indicating that the treatments prevented mitochondrial dysfunction. We also confirmed that complex I of the mitochondrial electron transport chain is a pharmacologic target of amobarbital in chondrocytes (Figure 4). In addition, blood tests showed that liver and kidney function were normal in all treatment groups, indicating that the drugs were not systemically toxic (Figure 5). Collectively these findings provide a sound basis for an IND filing with the FDA, and for planning clinical trials.

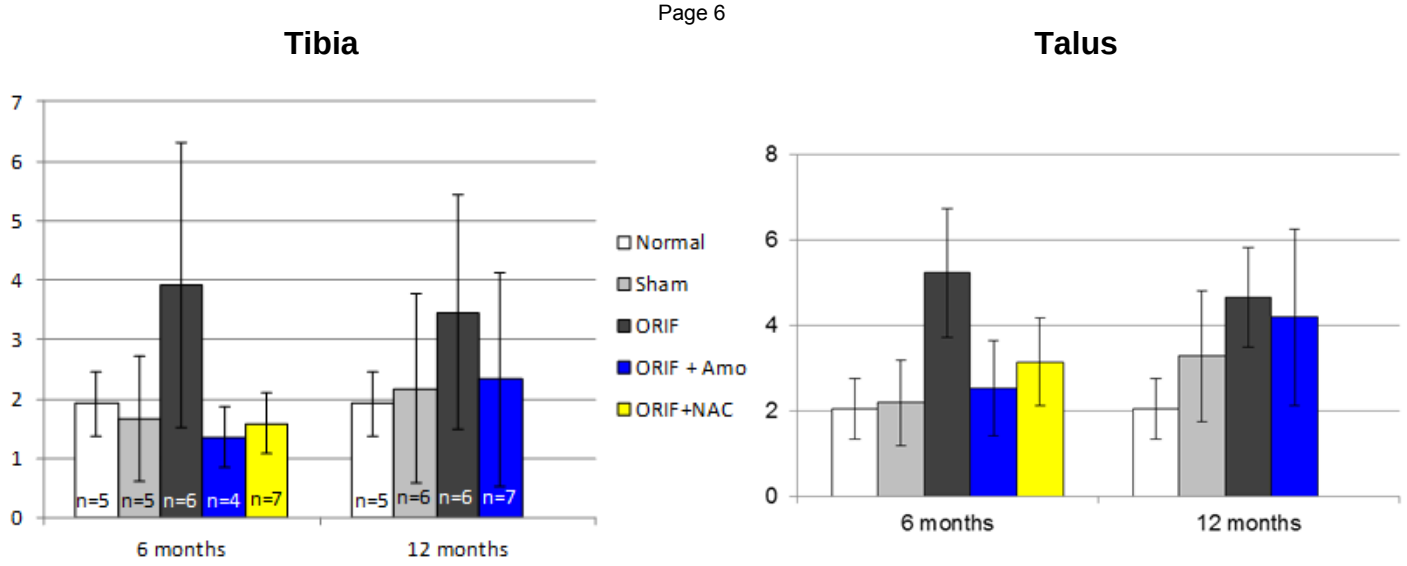

Figure 1. Mankin scores at 6 months and 12 months post-op. The results are for the distal tibia (left panel) and talus (right panel) from normal, sham-operated, untreated fractured joints (ORIF), and fractured joints treated with amobarbital (ORIF+Amo) or n-acetylcysteine (OIRF+NAC). The Sham, Amo and NAC groups scored significantly lower than control at 6 months in both compartments ($p < 0.05$).

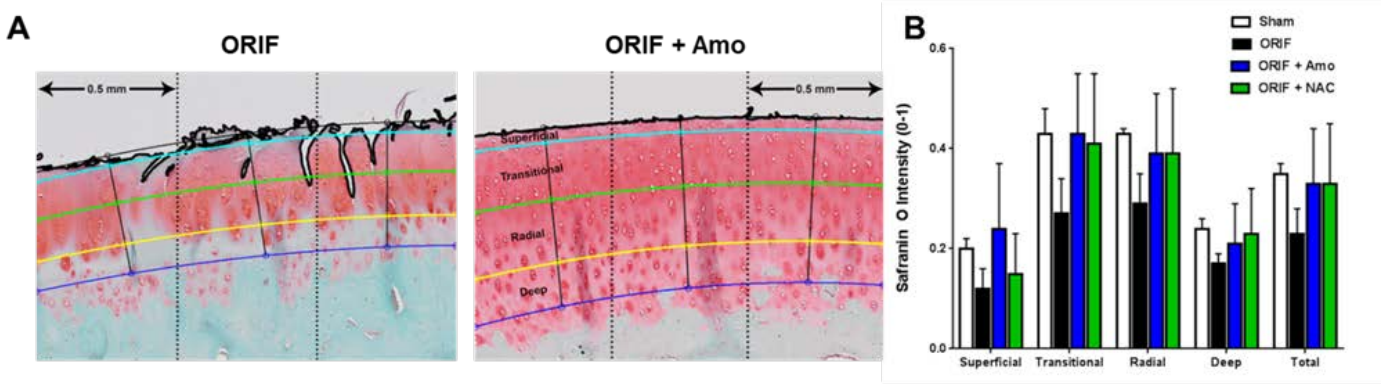

Figure 2. Treatment effects on the zonal distribution of proteoglycans. (A) The automated Mankin program automatically proscribed superficial, transitional, and radial zones in a non-treated specimen (ORIF), and a specimen treated with amobarbital (ORIF+Amo). (B) Safranin-O staining intensity (proportional to proteoglycan density), was measured in each zone. Amobarbital and NAC ameliorated proteoglycan losses induced by fracture, particularly in the transitional and radial zones. This implies that treatments maintained healthy biosynthetic activity, an outcome that is consistent with mitochondrial preservation.

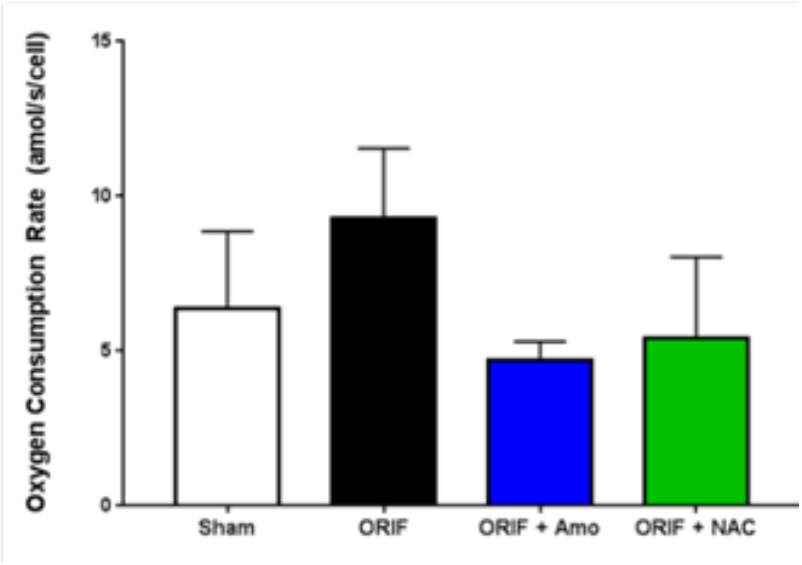

Figure 3. Amobarbital and NAC prevent osteoarthritis-related increases in respiration rate. Basal oxygen consumption rate (OCR) was measured in talar chondrocytes isolated at 6 months post-op from sham-operated controls, from fractured joints that were not treated (ORIF), and from fractured joints treated with amobarbital (ORIF+Amo) or NAC (ORIF+NAC).

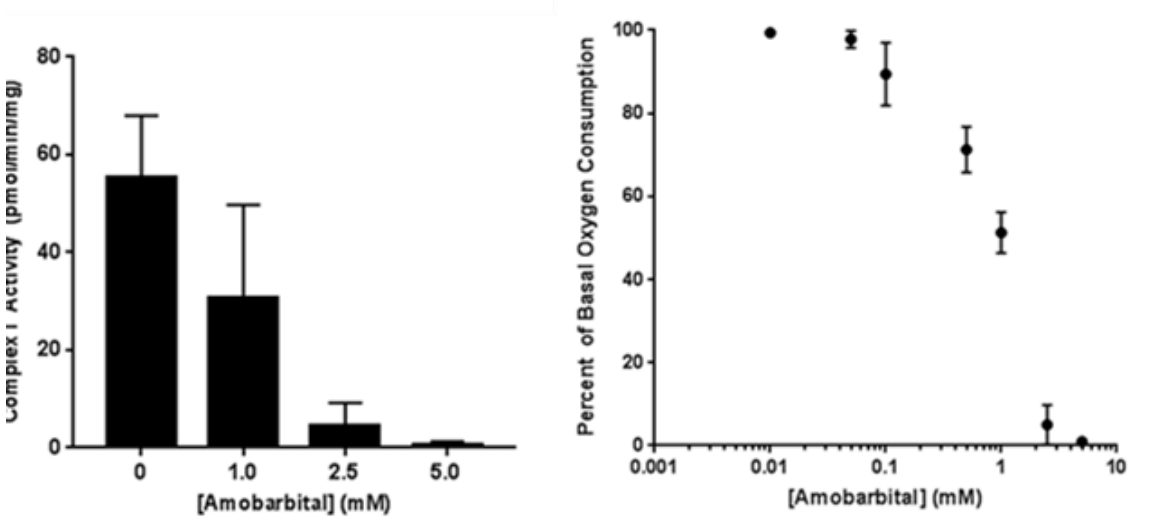

Figure 4. Amobarbital blocks respiratory and complex I activity in chondrocytes. Amobarbital at 2.5 mM inhibits greater than 90% of respiratory and complex I activity.

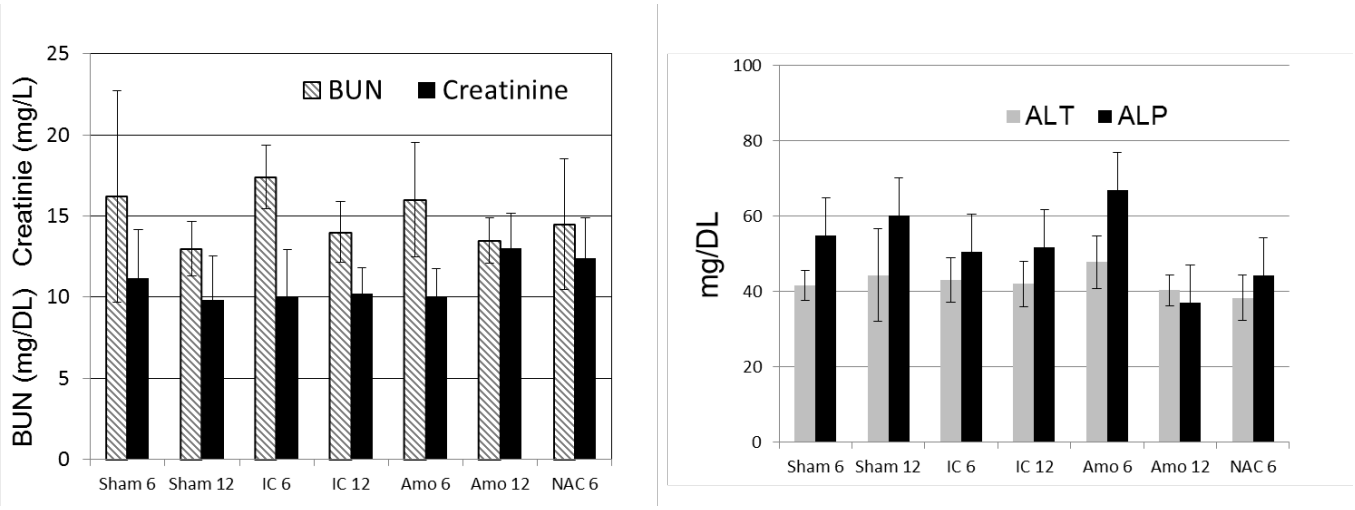

Figure 5. Blood chemistries. Results are shown for shams, untreated control (IC), amobarbital-treated (Amo) or NAC-treated at 6 or12 months post-op. Neither amobarbital nor NAC had significant effects renal function (BUN, Creatinine) or liver function (ALT, ALP).

4. Key Research Accomplishments

- Proved that PTOA in fractured joints can be forestalled by prompt intervention with NAC and amobarbital.
- Showed that NAC and amobarbital protect chondrocytes and chondrocyte mitochondria from oxidative damage and stress
- Demonstrated that mitochondrial preservation is associated with healthy levels of matrix synthesis
- Confirmed that therapeutic levels of NAC and amobarbital are non-toxic

5. Reportable outcomes

Publications, Abstracts, and Presentations

Mitchell C. Coleman, Jessica E. Goetz, Marc J. Brouillette, Michael C. Willey, Dongrim Seol, Emily B. Petersen, Behnoush Khorsand, Aliasger K. Salem, Douglas C. Fredericks, Todd O. McKinley, James A. Martin. *Complex I Inhibition after Intra-articular Fracture Prevents Rapid Progression of Osteoarthritis in a Porcine Model.* Accepted Abstract 2017 Orthopedic Research Society Meeting

Inventions, Patents and Licenses

Provisional patent covering intra-articular delivery of amobarbital to prevent PTOA (A PREVENTIVE THERAPY FOR POST-TRAUMATIC OSTEOARTHRITIS) was filed on behalf of Dr. Martin and colleagues by the University of Iowa Research Foundation in August 2015. The application states the following: “This invention was made with government support under grant number W81XWH-11-1-0583 awarded by the Department of Defense. The government has certain rights in the invention”. (please see appendix)

6. Other Achievements

A side experiment funded from other sources showed that amobarbital did not suppress acute synovitis in the rabbit impact model, supporting the hypothesis that the therapeutic value of amobarbital does not depend on anti-inflammatory activity.

7. Conclusions

These studies establish that the development of PTOA in fractured joints can be delayed by acute treatment with drugs that block a chondrocyte mechanotransduction pathway.

8. Future Activities

A final manuscript based on the findings will be submitted for publication in Nature Medicine by February 2017. The data from the project are sufficient to begin the process of applying for FDA approval to initiate a clinical trial.

9. References

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6. Brouillette MJ, Ramakrishnan PS, Wagner VM, Sauter EE, Journot BJ, McKinley TO, Martin JA. Strain-dependent oxidant release in articular cartilage originates from mitochondria. Biomech Model Mechanobiol. 2014 Jun;13(3):565-572. PMID: 23896937 PMCID: PMC3940668

10. Appendices

Please find attached:

1. Updated Quad Chart.

2. ORS 2017 Accepted Abstract: Coleman et al. *Complex I Inhibition after Intra-articular Fracture Prevents Rapid Progression of Osteoarthritis in a Porcine Model*

3. OARSI 2017 Submitted Abstract: Coleman et al. *Complex I Inhibition after Intra-articular Fracture Prevents Rapid Progression of Osteoarthritis in a Porcine Model*

4. PCT application: Preventative Therapy for Post-traumatic Osteoarthritis

Mitochondrial-Based Treatments that Prevent Post-Traumatic Osteoarthritis in a Translational Large Animal Intraarticular Fracture Survival Model

EWOF Year 1 Technical Progress Report: W81XWH-11-1-0583

PI: James A. Martin **Org:** The University of Iowa **Award Amount:** $2,559,912 (original), $2,559,259 (modified, incorporated by reference only according to award documents signed 17 Dec 2013)

Study/Product Aim(s)

- Aim 1: Measure the effects of free radical scavengers and inhibitors of electron transport on chondrocyte viability and metabolism and PTOA in a survival rabbit model .
- Aim 2: Determine the effects of compounds that dissolve filamentous actin and microtubulin in the rabbit model.
- Aim 3: Determine the efficacy of mitochondrial-based treatments on preventing PTOA in a large animal IAF survival model.

Approach

During this 1st extension without additional funds (EWOF), automated Mankin analysis of safranin-O stained sections was completed and statistical analyses were finalized. Abstracts describing the results were submitted to the Orthopedic Research Society and Osteoarthritis Research Society International. A manuscript has been drafted and formatted for submission to Nature Medicine (*Mitochondrial Responses to Intraarticular Fracture are a Disease-Modifying Target for Post-Traumatic Osteoarthritis Prevention*. Coleman, Goetz, Brouillette, Seol, Willey, Petersen, Khorsand, Morris, Salem, Fredericks, McKinley, Martin).

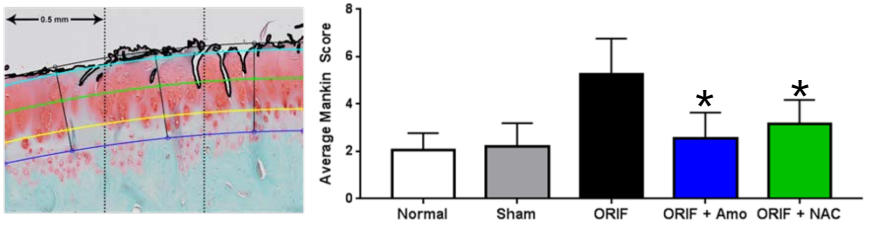

Figure. Completed histologic analysis and Mankin scores. The panel on the left shows an image after computerized processing to measure cartilage thickness, structure, cellularity, and proteoglycan density. The graph on the right shows total scores for the indicated groups (ORIF = open reduction and internal fixation after fracture). Amobarbital (Amo) and NAC significantly reduced scores compared to ORIF without treatment (* p = 0.01 and = 0.03 respectively).

Accomplishment: Data collection and analysis have been completed and a manuscript reporting the findings has been drafted.

Timeline and Cost

Activities	CY	12	13	14	15	EWOF1
(Aim 1)		Short-term tests (rabbit)				
(Aim 2)			Long-term tests (rabbit)			
(Aim 3)					Porcine tests	
Estimated Budget ($K)		**$380,245**	**$521,666**	**$819,756**	**$837,592**	
Actual Expenditures		$380,243	$483,511	$745,939	$856,613	$80,353

Updated: January 5 2017

Goals/Milestones

CY12-13 Goals – Specific Aim 1
☑ Obtain results for short-term rabbit (and pig) models
CY13-14 Goals – Specific Aim 2
☑ Begin histological processing on long-term rabbit models using amobarbital and NAC in hydrogel, single injection
CY14-15 Goal – Specific Aim 3
☑ Begin long-term pig IAF model first doing 6-month sham and injured control animals, then treatment animals
☑ Complete surgery on all IAF pig model animals
CY15 Goal – Wrap Up Project
☐ Finish histology on all remaining long-term rabbit and all IAF pig models, data analysis, and publish results

Budget Expenditure to Date (through August 31, 2016)
Projected Expenditure: $2,559,912 (expected to be spent before EWOF1).
Actual Expenditure: $2,546,658.21

Complex I Inhibition after Intra-articular Fracture Prevents Rapid Progression of Osteoarthritis in a Porcine Model

Mitchell C. Coleman[1], Jessica E. Goetz[1], Marc J. Brouillette[1], Michael C. Willey[1], Dongrim Seol[1], Emily B. Petersen[1], Behnoush Khorsand[1], Aliasger K. Salem[1], Douglas C. Fredericks[1], Todd O. McKinley[2], James A. Martin[1]
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Disclosures: Mitchell C. Coleman(N), Jessica E. Goetz(N), Marc J. Brouillette(N), Michael C. Willey(N), Dongrim Seol(N), Emily B. Petersen(N), Behnoush Khorsand(N), Aliasger K. Salem(N), Douglas C. Fredericks(N), Todd O. McKinley(N), James A. Martin(N).

INTRODUCTION: The rapid progression of PTOA after severe injuries like intra-articular fractures (IAF) combined with a lack of therapeutic options other than total joint replacement at end-stage suggests a need for new treatment paradigms to avoid or delay PTOA progression (1). Our previous *in vitro* studies have demonstrated protection of chondrocytes after impact or high strain using rotenone, a well characterized irreversible inhibitor of complex I of the mitochondrial electron transport chain (2, 3). Similar protective effects with the non-specific thiol antioxidant N-acetylcysteine (NAC) suggested that thiol oxidation and oxidative stress could also mediate injury in this pathway (4). This study extends our line of experimentation using a maximally clinically realistic IAF model (5) and the barbiturate amobarbital in place of rotenone. Amobarbital represents a more therapeutically viable inhibitor of complex I than rotenone given its reversible binding to complex I and decreased toxicity (6). **Hypothesis:** Administration of amobarbital or NAC subsequent to IAF and surgical fixation will delay the rapid progression of PTOA.

METHODS: Our porcine model utilizes a 40 J impact to the talus to cause a reproducible distal tibial fracture without surgical disruption of the joint capsule (4). Fractures are repaired using human-like open reduction and internal fixation (ORIF). PTOA occurs by six months, with lesions concentrated around the central-to-anterior portion of the medial talus. Either 2.5 mM amobarbital or 10 mM NAC (buffered with sodium bicarbonate) was dissolved in F-127/hyaluronic acid reverse thermal hydrogel. ORIF with vehicle alone (n = 5), amobarbital (n = 5), or NAC (n = 6) hydrogel was injected intra-articularly immediately after completion of the ORIF and again one week later. In addition to the injection, some NAC animals received placement of a small grain of poly(lactic-co-glycolic) acid (PLGA) encapsulated NAC for extended release over ~2 weeks after injury; however, we have pooled all NAC-receiving animals after observing no differences with the extended release pellet. Sham animals (n=5) receiving surgical procedures without fracture impact as well as naïve controls (Normal - n=5) were included. After treatment, animals were put into pasture for the duration of six months, after which they were euthanized. All procedures were conducted under IACUC approved protocols. Fractured and intact hocks were harvested and small pieces of cartilage were taken for live cell respiratory analyses. The remaining distal tibia and talar dome were fixed in formalin, decalcified and embedded in paraffin. Sagittal sections of weight-bearing tissue 5 µm-thick were cut and stained with safranin O, fast green, and Weigert's hematoxylin (5). Semi-automated Mankin scoring of these sections used a custom Matlab program, which quantified joint histology as previously described (5). Treatment effects were analyzed by one-way ANOVA.

Confirmation of inhibition of complex I by amobarbital was conducted *in vitro* using fresh harvested bovine chondrocytes (7). Briefly, cell lysate is added to phosphate buffer pH 7.2 with excess NADH, CoQ and complex III blockade while oxidation of NADH is monitored at 340 nm for 3 minutes with and without rotenone. This yields a rate of rotenone-inhibitable NADH oxidation, i.e. complex I activity. Complexes II, III, and IV were assessed with similar methods (7) but with no observable inhibition by amobarbital. We also conducted extracellular flux measurements via Agilent Seahorse XF96 in the presence and absence of 2.5 mM amobarbital, confirming > 90% decreases in oxygen consumption. In order to confirm the antioxidant effects of NAC one week after porcine IAF, steady states of the glutathione (GSH):glutathione disulfide (GSSG) redox couple were assessed as described (8).

RESULTS: Fresh whole chondrocyte lysates demonstrated a dose responsive inhibition of complex I by amobarbital, reaching >90% at maximal doses (**Figure 1A**, $p < 0.01$ at 2.5 mM, n = 3). Porcine tissue harvested 1 week after IAF demonstrated increased steady state percentages of GSSG, indicating oxidative stress, that were not present after NAC treatment (**Figure 1B**, $p < 0.01$ for Hydrogel v NAC, n = 6). These results support the hypotheses that amobarbital and NAC suppress complex I activity and oxidative stress, respectively. Sham animals displayed no increases in Mankin scores (**Figure 2A**) while fracture plus ORIF induced a significant increase in overall Mankin score (**Figure 2A**), decreased overall cartilage thickness and safranin O staining, and caused focal areas of eburnation (**Figure 2B,** representative micrograph). Animals receiving amobarbital after ORIF (ORIF+Amo) or NAC after ORIF (ORIF+NAC) showed statistically significant decreases in semi-automated Mankin scoring (**Figure 2A**, $p < 0.01$ for amobarbital, $p = 0.0322$ for NAC). These animals also clearly demonstrate thicker tissue and stronger staining for safranin O (**Figure 2B**, representative micrographs).

DISCUSSION: These results demonstrate that manipulation of joint mitochondrial metabolism after traumatic injury represents a viable pathway to treating PTOA. We have confirmed that the doses of amobarbital used *in vivo* provide inhibition of complex I directly. NAC at these doses and in comparable IAF pigs at one week provided protection against intracellular thiol oxidation, supporting the hypothesis that thiol-mediated oxidative stress plays a role in responding to the mitochondrial oxidant production inhibited by amobarbital. It is important to note that no porcine *in vivo* dose optimization or timing optimization studies have been done, but preliminary experiments in a rabbit impact model suggest the window for amobarbital treatment is likely between 4 and 8 hours after injury which represents a realistic window in which to provide a patient with an intra-articular therapy. We also have not yet combined this approach with any more specific anti-inflammatory approaches that may augment any efficacy.

SIGNIFICANCE: These results support the hypothesis that a therapeutic window for blunting PTOA exists at the earliest stages of disease. Given that NAC and amobarbital have such disparate effects upon cells but similar benefits for disease, these data also strongly support the importance of mitochondrial oxidant production and responsive intracellular thiol pathways in PTOA initiation after mechanical injury.

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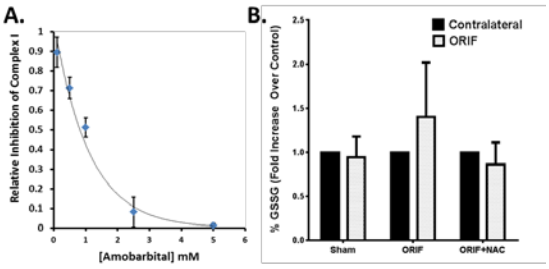

Figure 1. Amobarbital Inhibits Complex I Activity and NAC Prevents Intracellular Thiol Oxidation after IAF

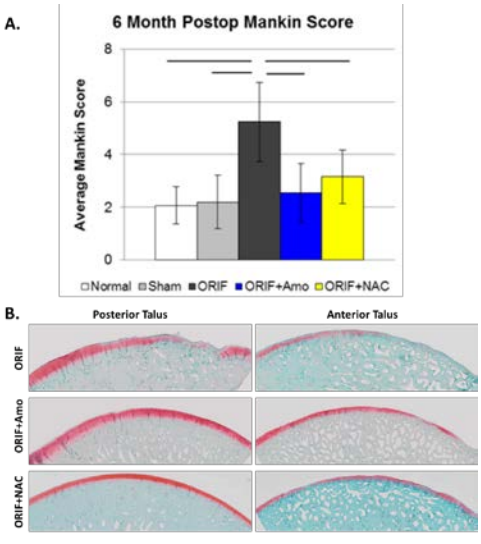

Figure 2. Amobarbital and NAC Prevent Rapid PTOA Progression Six Months Post-IAF

Complex I Inhibition after Intra-articular Fracture Prevents Rapid Progression of Osteoarthritis in a Porcine Model

Mitchell C. Coleman[1], Jessica E. Goetz[1], Marc J. Brouillette[1], Dongrim Seol[1], Michael C. Willey[1], Emily B. Petersen[1], Douglas C. Fredericks[1], Todd O. McKinley[2], James A. Martin[1]
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PURPOSE: The rapid progression of posttraumatic osteoarthritis (PTOA) after severe injuries like intra-articular fractures (IAF) creates an urgent need for preventive PTOA therapies that can extend healthy joint lifespans. Our previous studies demonstrated protection of chondrocytes after impact using rotenone, a well characterized irreversible inhibitor of complex I of the mitochondrial electron transport chain. Amobarbital represents a more therapeutically viable inhibitor of complex I than rotenone given its reversible binding and decreased toxicity. This study tests the hypothesis that rapid development of PTOA subsequent to IAF can be prevented by inhibiting mitochondrial metabolism after injury.
METHODS: Our minipig model utilizes a 40 J impact to the talus to cause a distal tibial fracture without surgical disruption of the joint capsule. Fractures are repaired using human-like open reduction and internal fixation (ORIF). PTOA occurs by six months, with lesions concentrated around the central-to-anterior portion of the medial talus. Amobarbital (2.5 mM) was added to F-127/hyaluronic acid reverse thermal hydrogel. Hydrogel vehicle (n = 5) or amobarbital (n = 5) was injected intra-articularly after completion of the ORIF and again one week later. Sham animals (n=5) receiving surgical procedures without fracture impact as well as naïve controls (n=4) were included. After treatment, animals were put into pasture for six months then euthanized. All procedures were conducted under IACUC approved protocols. Fractured and contralateral hocks were harvested and the distal tibia and talar dome fixed in formalin, decalcified and embedded in paraffin. Sagittal sections of weight-bearing tissue 5 μm-thick were cut and stained with safranin O, fast green, and Weigert's hematoxylin. Semi-automated Mankin scoring was done using a custom built Matlab program. Treatment effects were analyzed by one-way ANOVA. Confirmation of inhibition of complex I by amobarbital was conducted using fresh bovine chondrocytes. Excess NADH, CoQ and complex III blockade were added to lysates while oxidation of NADH was monitored spectrophotometrically at 340 nm for 3 minutes with and without rotenone. This yields a rate of rotenone-inhibitable NADH oxidation, i.e. complex I activity. Complexes II, III, and IV were assessed with similar methods but with no inhibition by amobarbital.
RESULTS: Fresh whole chondrocyte lysates demonstrated a dose responsive inhibition of complex I by amobarbital, reaching >90% at maximal doses (**Figure 1**, $p < 0.01$ at 2.5 mM, $n = 3$). At 6 months, sham animals displayed no increases in talar Mankin scores (**Figure 2A**) while fracture (ORIF) induced a significant increase in Mankin score (**Figure 2A**), decreased overall cartilage thickness and safranin O staining, and caused focal areas of eburnation on the talus (**Figure 2B,** representative micrograph). Animals receiving amobarbital after ORIF (ORIF+Amo) showed statistically significant decreases in talar Mankin scoring (**Figure 2A**, $p < 0.01$) and thicker cartilage that stained more strongly for safranin O than fractured controls (**Figure 2B**, representative micrographs).
CONCLUSIONS: These results support the hypothesis that a therapeutic window for blunting PTOA with amobarbital exists at the earliest stages after IAF. This approach has not yet been combined with any anti-inflammatory approaches that may augment efficacy and no dose/timing optimization studies have been done. Manipulating joint mitochondrial metabolism after traumatic injury may represent a viable pathway to preventing PTOA.
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Inventions

No.	Title	Disclosure Date	Disclosure Status	Inventors	Invention License Officers
2015-131	A Novel Preventive Therapy for Post-Traumatic Osteoarthritis Focused on Acute Inhibition of Mitochondrial Electron Transport	May 21, 2015	Active	Todd O McKinley ☆ James A. Martin Mitchell Coleman Tae-Hong Lim Marc Brouillette	Mihaela D. Bojin

Agreements Directly Related to this Intellectual Property

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